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DEVELOPMENTAL BIOLOGY

Developmental Biology 256 (2003) 258–275

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Cyclin B synthesis is required for sea urchin oocyte maturation

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Received for publication 19 September 2002, revised 20 December 2002, accepted 20 December 2002

Abstract

Sea urchins are members of a limited group of animals in which meiotic maturation of oocytes is completed prior to fertilization. This is different from oocytes of most animals such as mammals and amphibians in which fertilization reactivates an arrested meiotic cycle. Using a recently developed technique for in vitro maturation of sea urchin oocytes, we analyzed the role of cyclin B, the regulatory component of maturation-promoting factor, in the control of sea urchin oocyte meiotic induction and progression. Oocytes of the sea urchin *Lytechinus variegatus* accumulate significant amounts of cyclin B mRNA and protein during oogenesis. We analyzed cyclin B synthetic requirements in oocytes and early embryos by inhibiting cyclin B synthesis with DNA and morpholino antisense oligonucleotides. Cyclin B synthesis is not necessary for the entry of G2-arrested oocytes into meiosis; however, it is required for the proper progression through meiotic divisions. Surprisingly, mature sea urchin eggs contain significant cyclin B protein following meiosis that serves as a maternal store for early cleavage divisions. We also find that cyclin A can functionally substitute for cyclin B in early embryos but not in oocytes. These studies provide a foundation for understanding the mechanism of meiotic maturation independent of the zygotic cell cycle.

Keywords: Meiosis; Egg; Cyclin B; MPF, Embryo

Introduction

Cyclins and their catalytic kinase partners (cdks, or cyclin-dependent kinases) are well recognized as stimulators of cell-cycle progression (reviewed in Pines, 1999). These kinases are only active when bound to their regulatory subunits, cyclins. Cyclin-dependent kinases preferentially complex with particular cyclins and are required only at specific times during mitotic progression. Although levels of cdks remain constant, the levels of cyclins usually fluctuate during the cell cycle due to periodic synthesis and degradation, resulting in transient kinase activities. Cyclins are classified as G1-, S-, or M-phase regulators based on when their activities are required in the cell cycle (Pines, 1999).

Mitotic cyclins were initially discovered in clams and sea urchins as rapidly synthesized proteins that were degraded at mitosis during the first cell cycle (Evans et al., 1983). Consistent with their classification, the levels of newly synthesized mitotic cyclins peak in M-phase, and mitotic cyclin/cdk1 (also referred to as cdc2) complexes govern the transition to, and progression through, M-phase (reviewed in Pines, 1999). Mitotic cyclins include three distinct gene families of cyclins A, B, and B3 (Jacobs et al., 1998) that appear to have overlapping functional properties. In *Drosophila* embryos, when one of the mitotic cyclins is removed experimentally, the remaining mitotic cyclins can compensate and support normal cell-cycle progression (Jacobs et al., 1998; Knoblich and Lehner, 1993). Cyclin B-cdk1 kinase, for example, phosphorylates numerous substrates, including nuclear lamins, histone H1, transcription factors, and cytoskeletal regulators, causing structural changes in the nuclear envelope, chromatin, and cytoskeleton (reviewed in Nigg, 1995).

The mitotic cell cycle is modified in a meiotic cell cycle, which is essential for gamete formation. In females, primor-

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doi:10.1016/S0012-1606(02)00134-3

dial germ cells go through several rounds of mitosis before differentiating into oocytes and entering meiosis. In most of the species studied, the oocyte first replicates its DNA and then arrests in prophase of meiosis (which is essentially a G2/M transition) where it spends the greater part of its life growing independent of further cell-cycle progression. Late in the oocyte's growth period, it becomes responsive to a mitogen signal, which induces the progression of the meiotic cycle to the first or second metaphase where it arrests again (reviewed in Masui and Clarke, 1979; Taieb et al., 1997). Fertilization in most species is the final stimulus for meiotic completion and entry of the male pronucleus occurs as the female nucleus completes meiotic reduction.

A key point in the G2/M transition during meiotic resumption in oocytes is activation of maturation promoting factor (MPF[1]) (Taieb et al., 1997). MPF was identified in, and subsequently purified from, mature eggs of *Xenopus* (Lohka et al., 1988; Masui and Markert, 1971). It consists of cyclin B (or B1 and B2 in vertebrates) and cdk1 kinase as its catalytic partner (reviewed in Maller, 1990), although cyclin A also forms active complexes with cdk1 (Draetta et al., 1989). To proceed through meiotic divisions, the oocyte accumulates and then activates MPF. During meiosis, the exit from MI and entry into MII are caused by the inactivation and reactivation, respectively, of MPF. However, regulation of MPF during this period is different than that in the mitotic cell cycle, namely, MPF activity increases rapidly after the first meiotic division, and the second M-phase starts without a preceding S-phase. The cell-cycle dynamics of cyclin A, cyclin B, and cdk1 kinase during oocyte maturation have been studied in many organisms (reviewed in Kishimoto, 1999; Yamashita, 1998), and the significant differences found between species argue against a unified mechanism regulating MPF activity in oocytes (Taieb et al., 1997).

All oocytes that have been studied contain cdk1 levels that do not change appreciably at the time of meiotic entry (Choi et al., 1991; Draetta et al., 1989; Katsu et al., 1993; Kobayashi et al., 1991). However, protein levels of cyclins in a number of species have been found to change before and during maturation (see below). Furthermore, synthetic requirements for cyclin B at the time of oocytes' entry into meiosis also differ between animals. One extreme mechanism of cyclin B regulation is seen in fishes and anuran amphibians (except *Xenopus*), where full-grown immature oocytes do not contain cyclins B1 and B2. After hormonal stimulation of these oocytes, cyclin B is synthesized from stored RNA, binds to preexisting cdk1, and becomes active MPF (reviewed in Yamashita, 1998). We now know that the inhibitory phosphorylation of cdk1 (on Tyr15) is absent in these oocytes, and therefore, the MPF activity is determined only by cyclin B protein levels. In contrast, full-grown oocytes of *Xenopus* contain significant amounts of cyclin B2 and B5 protein, and additional cyclin synthesis (of either cyclin B1, 2, 4, or 5) is not required for the activation of MPF and progression into meiosis (Hochegger et al., 2001).

Cyclin B synthetic requirements have not been studied directly in mammalian oocytes, but have been inferred from inhibition of total protein synthesis. In mouse oocytes, protein synthesis is not necessary for GVBD (de Vant'ery et al., 1996), while oocytes of other mammalian species, such as goat, pig, and cow, do not activate cdk1 kinase in the absence of protein synthesis (Le Gal et al., 1992; Naito et al., 1995). Therefore, despite the conservation of MPF structure and function, significant variations exist between animals in the mechanism of MPF activation bringing about oocyte meiotic maturation.

Although sea urchin eggs have been a popular choice for studies of fertilization, the oocyte has been much less tractable and no studies have focused on the regulation of its meiosis. When a sea urchin oocyte is stimulated to proceed through meiotic maturation, it is released from a G2 arrest and progresses through two sequential meiotic divisions, resulting in a mature fertilizable egg with a haploid pronucleus. This haploid state lasts for weeks until fertilization and is thought to be a G0- or G1-like state of the cell cycle. This is in contrast to most animals, where the oocytes do not complete meiosis prior to fertilization, but are arrested at a second point during meiosis, and await fertilization for the arrest to be relieved. This arrest may occur in MI (insects and ascidians) or in MII (most vertebrates) (Masui and Clarke, 1979). Given the remarkable diversity of ways in which cyclin B is regulated within such phyla as amphibians and mammals, we set out to study cyclin B and MPF regulation in this unusual oocyte where the meiotic and embryonic cell cycles are independent of one another.

Materials and methods

Reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Animals

Adult *Lytechinus variegatus* were obtained from the Duke University Marine Laboratory (Beaufort, NC) or collected in Tampa, FL. Females were shed by KCl (0.5 M) injection and ovaries were then removed and minced in artificial sea water (ASW; Coral Life Scientific Grade Marine Salt; Energy Savers Unlimited, Inc., Carson, CA). Oocytes and eggs were isolated and cultured in ASW at 22°C. To obtain embryos, the eggs were fertilized and cultured at 22°C. ASW was supplemented with 1 mM 3-amino-triazol to prevent hardening of the fertilization envelope when

[1] Abbreviations used: AS, antisense; ASWT, artificial sea water with added 0.05% Tween 20; GVBD, germinal vesicle breakdown; MO, morpholino oligonucleotide; MPF, maturation/mitosis promoting factor; ODN, oligodeoxynucleotide; TBST, Tris-buffered saline containing 0.05% Tween 20.

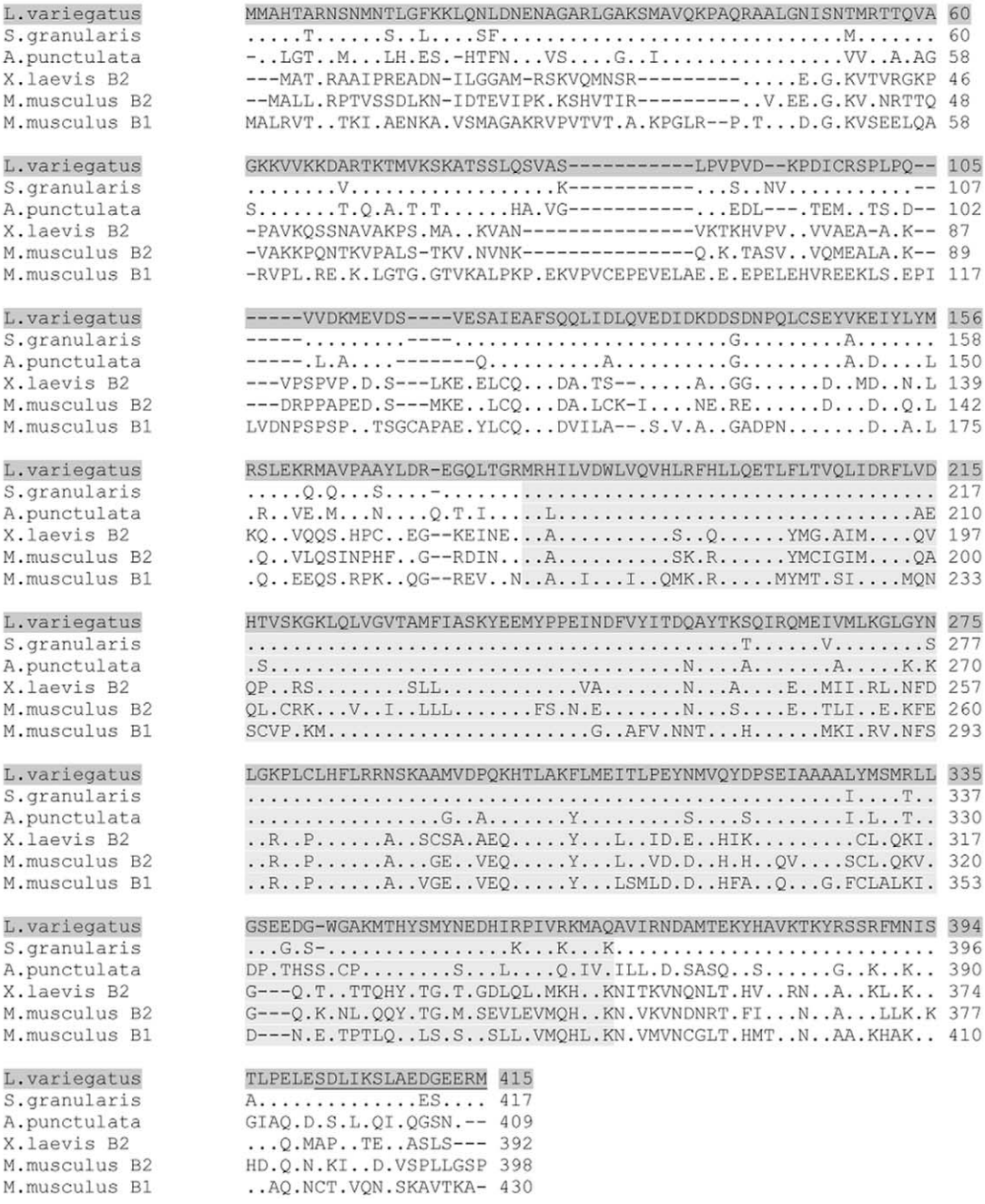

Fig. 1. Alignment of three sea urchin, *Xenopus*, and mouse cyclin B sequences. The alignment was performed with ClustalW program (Thompson et al., 1994). Dots represent amino acids identical to *L. variegatus* cyclin B sequence (dark gray). The cyclin box sequence is highlighted in light gray (according to Simple Modular Architecture Research Tool, or SMART: Schultz et al., 2000). The peptide used as the antigen is underlined. The sequences used in the alignment are *L. variegatus* (this work), *S. granularis* (Lozano et al., 1998), *A. punctulata* (Pines and Hunt, 1987), *X. laevis* cyclin B2 (Minshull et al., 1989), *M. musculus* cyclin B2 (Chapman and Wolgemuth, 1993), *M. musculus* cyclin B1 (Chapman and Wolgemuth, 1992).

embryos were cultured for collection of protein samples or for immunolocalizations.

Cloning of the cDNA

The *L. variegatus* ovary λZAP cDNA library (gift of Dr. David R. McClay, Duke University; Wessel et al., 1998) was screened by plaque-lift hybridization, using a labeled cyclin box fragment of *Strongylocentrotus purpuratus* cyclin B as a heterologous probe. A single positive phage was obtained, containing a 2524-bp insert that encoded the full-length cyclin B protein (Fig. 1). DNA sequencing was performed by the macromolecular sequencing facility at Brown University using an ABI 377 prism-automated sequencer (Perkin–Elmer, Foster City, CA). The majority of sequences reported here is based on this single clone, but 5′

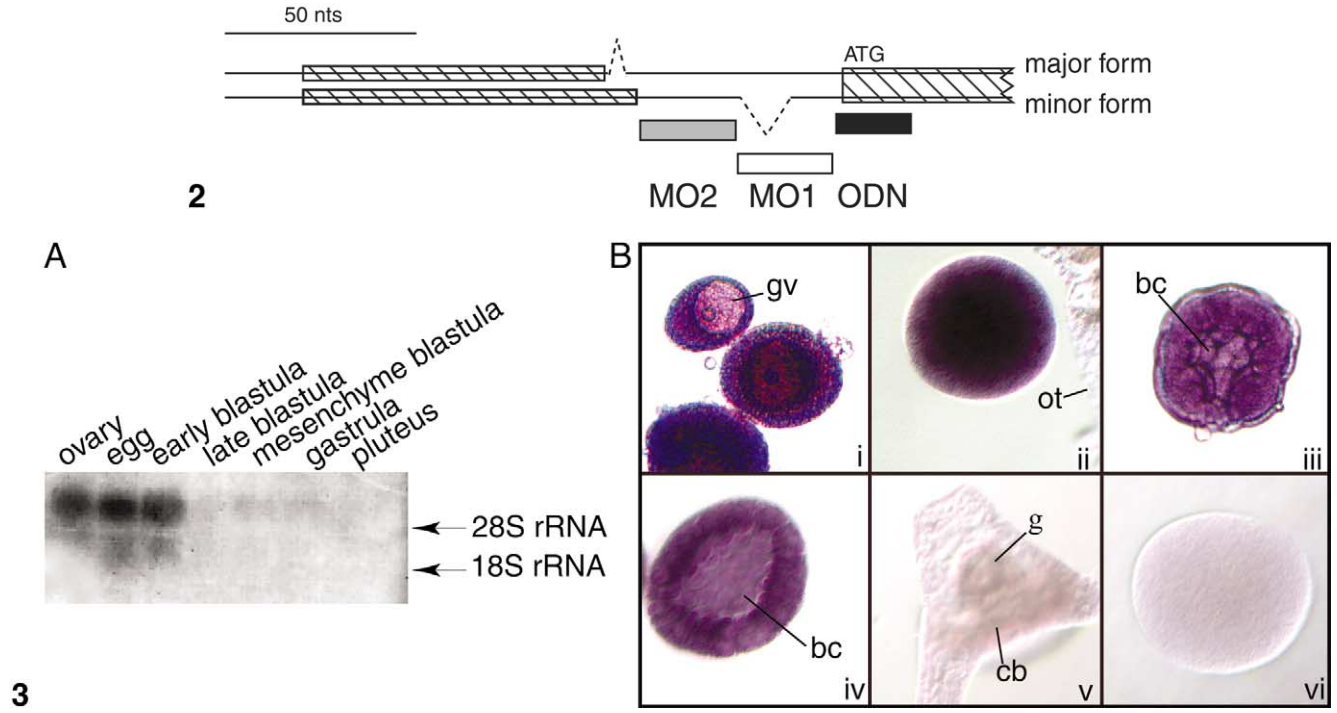

Fig. 2. Two forms of *L. variegatus* cyclin B mRNA have been identified, with variations in the 5′UTR. The major and minor forms differ from one another by small (5–12 bp) insertions. The schematic shows positions of these insertions relative to each other, to upstream putative ORFs, and to the initiating methionine. ATG, initiator codon; ODN, the region to which DNA antisense and sense oligos were designed; MO1; morpholino 1; MO2; morpholino 2. The striped boxes represent positions of upstream putative ORFs (MHLASSFILKQKAEAENNLDSTFSRRL in major splice variant, MHLASSFILKQKAE-AENNLDSTFSRRLYYSH in minor splice variant) and cyclin B ORF. The abundance of the forms was estimated based on their frequency in the 5′RACE produced clones. The reported sequence of the phage clone of cyclin B belongs to the "major" isoform.

Fig. 3. (A) Accumulation of *L. variegatus* cyclin B mRNA. Ten micrograms of *L. variegatus* total RNA from the indicated developmental stages was resolved by gel electrophoresis. The filter was then hybridized to the cyclin B ORF probe. Cyclin B transcript (~5 kb in length) is abundant in ovary, eggs, and early blastulas. The transcripts are significantly less abundant in late and mesenchyme blastulas, gastrulas, and plutei. Arrows show the positions of 28S and 18S rRNAs. (B) RNA *in situ* hybridization of *L. variegatus* embryos with cyclin B RNA probe. (i) Cyclin B mRNA is enriched in the cytoplasm of the oocytes and (ii) eggs, and persists through 32-cell and blastula stage embryos (iii, iv). No label is present in ovarian tissue fragment shown in ii. (v) Cyclin B mRNA amounts decrease in pluteus stage larva. A low signal is detected in the gut and ciliary band. (vi) Negative control, sense cyclin B RNA used as a probe does not generate any signal in the egg. gv, germinal vesicle, the nucleus of an oocyte; ot, ovarian tissue; bc, blastocoel; g, gut; cb, ciliary band.

and 3′ PCR screens suggest that the clone is representative of the cyclin B present in sea urchin oocytes. The sequence of the clone was entered in GenBank (Accession No. AY227808). A different cDNA library was employed for further screening. This library was made in λZAP II (Stratagene, La Jolla, CA) prepared from poly(A)+ mRNA isolated from L. variegatus ovaries containing mixed stages of oocytes. The *L. variegatus* ovary λZAP II cDNA library was screened by nested PCR amplification with the gene-specific primers 5′-TTGTCACTGTCGTCTTTGTCG-3′ and 5′-GACGTAGCCTTGGATTTTACC-3′ for the 5′ screen and 5′-CAATGAAGATCACATCAGACC-3′ and 5′-GATGGCACAGGCTGTTATCAG-3′ for the 3′ screen in conjunction with vector primers M13 forward and reverse in the first of nested rounds, and T3 and T7 in the second nested round. The PCR conditions were the following: after initial denaturation for 5 min at 94°C, the reaction was subjected to 30 cycles of denaturation (94°C, 1 min), primer annealing (1 min), and extension (72°C, 1 min). The primer annealing temperature was 40°C in 5′-RACE both rounds, and 45 and 49°C in 3′-RACE first and second rounds, respectively. The amplification products were cloned into pGEMT-Easy (Promega, Madison, WI).

RNA analysis

Northern blot analysis of cyclin B mRNA expression during development was performed as described using 10 μg of total RNA from ovaries, eggs, and embryos at several developmental stages (LaFleur et al., 1998). ^{32}P-labeled cyclin B probe corresponding to nucleotides 157–1404 of GenBank entry was synthesized with RTS RadPrime DNA Labeling system (Gibco Life Technologies, Inc., Gaithersburg, MD) according to kit directions. The loading levels were ascertained by OD measurements of the samples, as well as by the intensity of ethidium bromide staining of rRNA bands. For in situ RNA hybridization of cyclin B mRNA, ovaries, eggs, and early stage embryos were fixed in 2% glutaraldehyde and prepared as described (Ransick et al., 1993). Digoxigenin-labeled antisense transcripts representing nucleotides 710–2524 of GenBank entry were synthesized with the Megascript kit (Ambion Inc., Austin, TX)

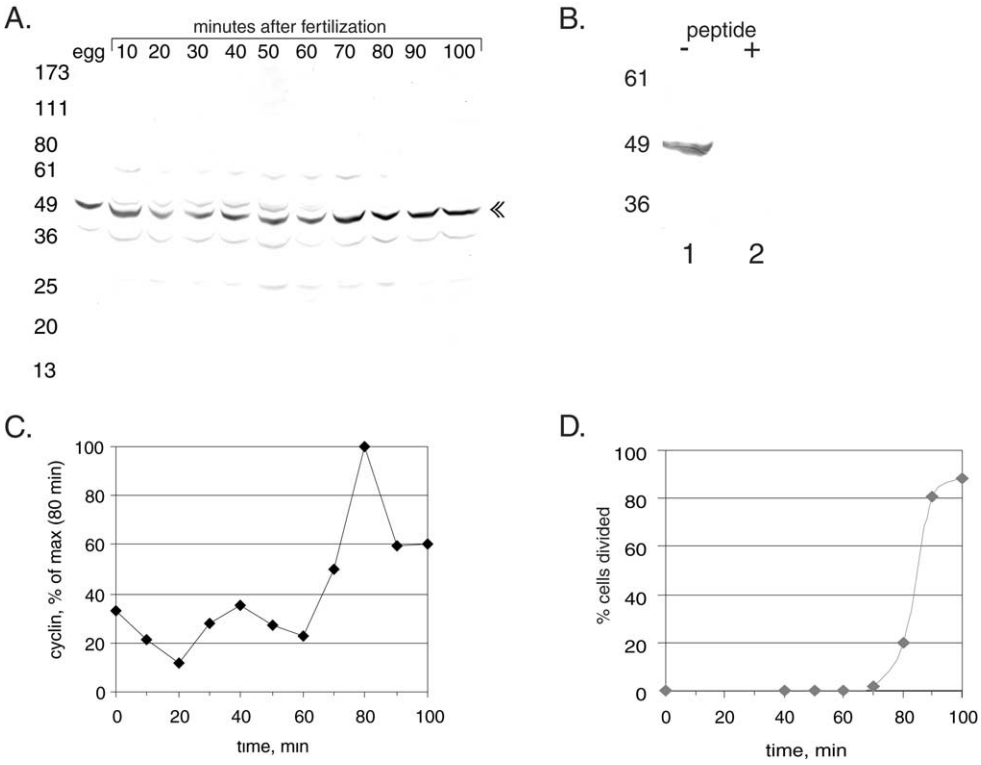

Fig. 4. Expression of cyclin B protein during the first cell cycle of sea urchin embryos. (A) A representative Western blot with anti-cyclin B antibodies is shown with the positions of molecular weight markers on the left of the gel. The lanes are unfertilized eggs followed by extracts prepared at indicated times after fertilization (25 μg/lane). Embryos reached the two-cell stage at 90 min (see also D). Arrowheads mark the position of major cyclin B band. The bands at 50 kDa appear to be phosphorylated products and the bands at 39 and 27 kDa may be breakdown products of cyclin B (data not shown). (B) Specificity of anti-cyclin B antibodies. Western blot of egg extract probed with anti-cyclin B (lane 1), or with anti-cyclin B that has been preincubated with 100 μM of peptide antigen (lane 2). + and − indicate presence and absence of antigen blocking, respectively. (C) Quantification of cyclin B bands (in A), normalized per protein staining intensity of the duplicate gel. Cyclin B levels peak at 80 min postfertilization. (D) Progression of the embryos through the first cell cycle, assessed under the microscope during sample acquisition for A.

according to the kit directions, from the plasmid linearized with *Sal*I using T3 RNA polymerase.

Immunological approaches

C-terminal cyclin B peptide (SDLIKSLAEDGEERM, with N-terminally added cysteine) synthesis, BSA coupling, and generation of rabbit polyclonal serum against the peptide was performed by Sigma Genosys (The Woodlands, TX). Antibodies were affinity-purified by linking the peptide immunogen to agarose beads (Pierce Sulfolink kit, Rockford, IL) and sequentially binding and eluting the antibody as per the manufacturer's protocol.

Electrophoresis and immunoblot analysis

Protein samples isolated from eggs and early embryos were subjected to methanol extraction (Chapdelaine et al., 2001) essentially as described and were then subjected to SDS–PAGE followed by immunoblot analysis. For each analysis, the protein pellet was resuspended in SDS–PAGE sample buffer and denatured for 5 min at 100°C. Equal amounts of proteins per lane (25 μg) were resolved on a 4–20% gradient gel (ICN Biomedicals Inc., Aurora, OH) and either stained with Coomassie blue or transferred to nitrocellulose for immunolabeling. Blots were preblocked by incubation in blotto (50 mM Tris–Cl, pH 7.5, 0.18 M NaCl, 0.05% Tween 20, 3% nonfat dry milk) for at least 15 min. Affinity-purified anti-cyclin B antibodies were diluted to 25.3 μg/ml in blotto and applied to the nitrocellulose for 1 h at room temperature. The blots were then washed in blotto three times over at least 20 min. The secondary alkaline phosphatase conjugated goat anti-rabbit IgG was diluted 1:30,000 in blotto and applied to the nitrocellulose for 1 h at room temperature. Blots were washed in blotto two more times and then washed in TBST once, each wash lasting at least 5 min. Signal detection was carried out with BCIP/NBT development. For peptide blocking experiments, the diluted antiserum was incubated for 1 h with 100 μM antigenic peptide and then cleared by centrifugation at 14,000 rpm for 10 min.

Immunolocalizations

Immunofluorescent localization was performed in whole mounts: after fixation in 4% paraformaldehyde in ASW, the

samples were extracted with methanol/EGTA and blocked 4 h to overnight in TBST supplemented with 3% nonfat dry milk. Affinity-purified polyclonal antibodies against cyclin B were diluted to 10.9 μg/ml in TBST/milk. The secondary antibody (Cy-3-conjugated affinity-purified goat anti-rabbit IgG; Jackson Research Laboratories, Westgrove, PA) was diluted 1:300 in TBST. For the immunostaining of morpholino-injected embryos, rhodamine-conjugated Fab fragments of goat anti-rabbit IgG (Jackson Research Laboratories) were employed. To detect DNA, the embryos were counterstained with 0.2 μg/ml Hoechst 33258 (Molecular Probes, Inc.). Microtubules were detected with anti-α-tubulin monoclonal antibodies (DM1A) diluted 1:500 and incubated overnight at 4°C, followed by secondary FITC-conjugated IgG (Jackson Research Laboratories) incubated for 1 h. Signals were visualized and recorded by laser-scanning microscopy with a Zeiss LSM 410 confocal microscope (Zeiss Inc., Thornwood, NY) with appropriate filters.

Quantification of cyclin B localization in situ

Equatorial confocal images of oocytes labeled with cyclin B antibodies were analyzed in Metamorph (Universal Imaging Corp., Downingtown, PA) to determine the oocytes' diameter and the ratio of intensity of cyclin B immunostaining in the nucleus to the intensity of this staining in the cytoplasm. The oocytes were then grouped according to their diameter into small ($<$75 μm), medium (75 to 100 μm), and full-grown ($>$100 μm). For each group, the percentage of oocytes exhibiting a ratio greater than 1 was determined.

Histone kinase assays

Histone H1 kinase assays were performed on immunoprecipitated samples as previously described (Schnackenberg and Marzluff, 2002; Sumerel et al., 2001). Antibodies to cyclin B were used for immunoprecipitation from sea urchin oocyte, egg, or embryo extracts at the indicated times after fertilization. Histone H1 (Roche Applied Science, Indianapolis, IN) was used as a substrate. Phosphorylated histone was resolved on SDS–PAGE and exposed to a phosphoimager plate, and the radioactivity associated with the histone bands was quantified using NIH Image software. Percentage contribution of the negative control to the specific signal was calculated for the 40 min value; the same percentage value was subtracted from the rest of values. The egg value was set to 1, and the rest were scaled accordingly.

Microinjections

Microinjections were performed as described (Conner and Wessel, 1998). Oocytes or fertilized embryos were placed in a Kiehart chamber in ASW and injected with appropriate solutions that never exceeded 5% of the cell volume. An oil droplet of dimethylpolysiloxane or a 0.1 mg/ml solution of fluorescent dextran (labeled with rhodamine or oregon green; Molecular Probes Inc.) was coinjected into cells as a marker. Both control and test reagents were usually injected into the same batch of cells, and the cells were then incubated at 22°C.

Oligonucleotides

The antisense oligonucleotide against sea urchin cyclin B mRNA used in the experiments had the following sequence: 5′-TTGCGGTATGAGCCATCATT-3′. The sense oligonucleotide used as a control was 5′-AATGATGGCTCATACCGCAA-3′. Both oligonucleotides included the initiation codon of the cyclin B open reading frame. The oligonucleotides were synthesized and desalted by Gibco Life Technologies, Inc. Concentrations of stock ODNs for injections were 1 or 5 mg/ml in dH_2O.

Morpholino

Morpholino oligonucleotides were produced by GeneTools, LLC (Philomath, OR). The sequences of morpholino oligonucleotides used were as follows: 5′-CAGTAGATTCAACTGCAAGTACAAC-3′ for MO1, 5′-CTGCCAAATTCTTCGGAATAGTCAA-3′ for MO2, 5′-CAACATGAACGTCAACTTAGATGAC-3′ for inverse control, and 5′-AAGCCATAGTTTTCAGTGTCTAAAT-3′ for cyclin A MO.

Synthesis and polyadenylation of cyclin B mRNA

The cyclin B ORF was directionally cloned into pBluescript plasmid containing β-globin 3′UTR (originally from a modified pSP64T vector; Wang et al., 1999). mRNA was synthesized with mMessage mMachine in vitro translation kit (Ambion) from the plasmid linearized with *Xho*I using T3 RNA polymerase according to manufacturer's protocol. The mRNA was polyadenylated with the PolyA Tailing Kit (Ambion) as per manufacturer's instructions.

In vitro oocyte maturation

In vitro maturation of oocytes was performed as reported previously (Berg and Wessel, 1997), with modifications. Oocytes were isolated from minced ovaries by mouth pipette and transferred to a 96-well Falcon flexible assay plate (Becton–Dickinson, Oxnard, CA) at 22°C, where they matured spontaneously. A subset of the plate wells contained 10 μM sense or antisense cyclin B oligonucleotides. The cells took up the oligonucleotides from the medium, as was also seen in mammalian tissue culture (reviewed in Dokka

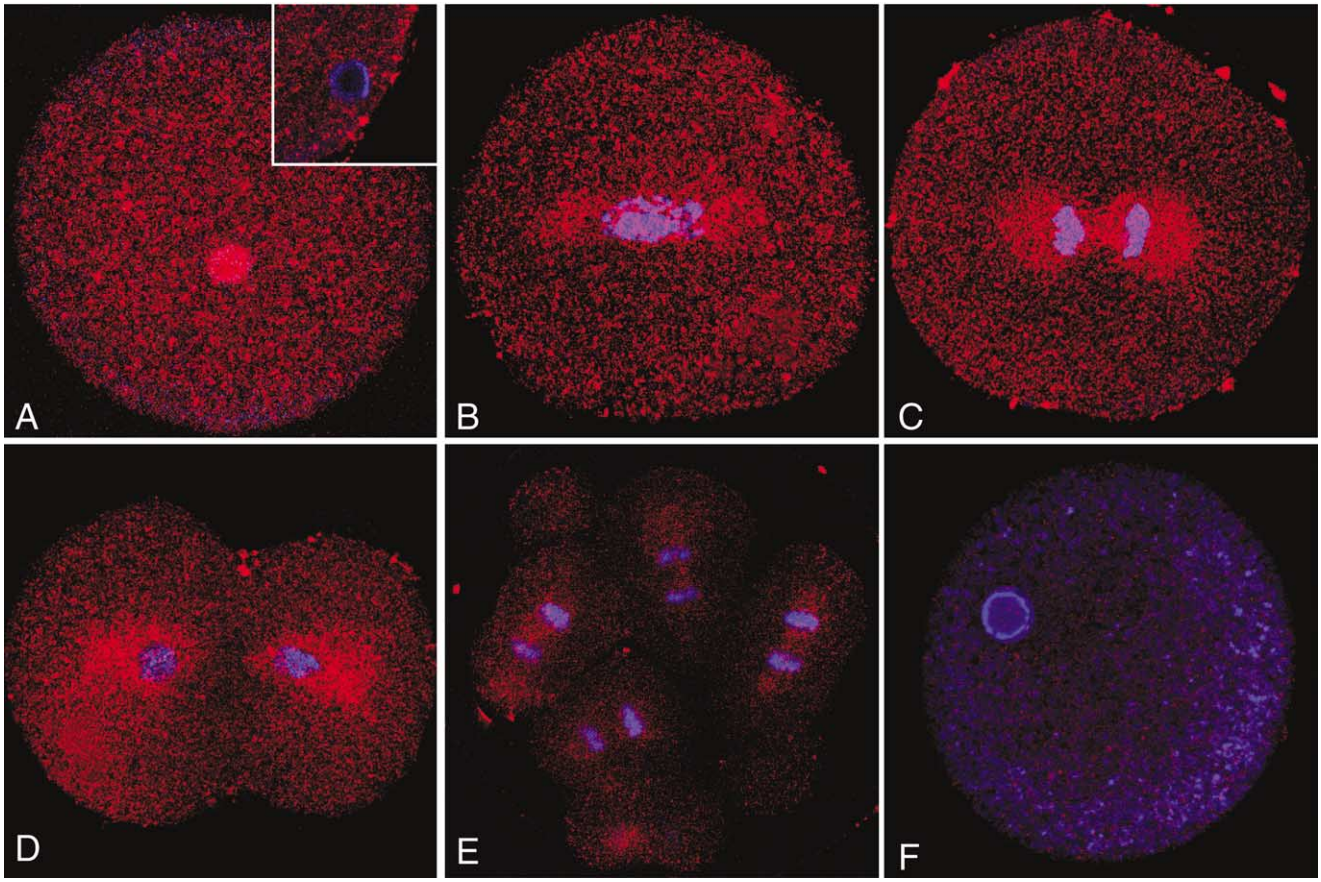

Fig. 5. Immunolocalizations with cyclin B antibody in embryos. Sea urchin embryos were fixed in paraformaldehyde, immunostained with anti-cyclin B, and counterstained with Hoechst DNA stain, as described under Materials and methods. (A) Egg; inset, cyclin B is excluded from the female pronucleus by 10 min postfertilization. (B–D) Embryos progressing through the first mitotic division: (B) metaphase; (C) anaphase/early telophase; (D) late telophase. (E) Embryo progressing through the fourth mitotic division. (F) Negative control: egg stained with the antibody preabsorbed with the antigenic peptide. The images were acquired by confocal microscopy. Specific signal is competed by preabsorption with the antigenic peptide.

and Rojanasakul, 2000). Microinjected oocytes were left to mature in the Kiehart chamber where they were injected.

Degenerate PCR to clone GAPDH

To generate an RT-PCR control, we cloned a region of the transcript encoding the metabolic enzyme GAPDH. The sequence was entered in GenBank (Accession No. AY227809). The degenerate primers used in the reaction were 5′-AAGGTCATCMAYGACAACTT-3′ and 5′-TCIAYIACICKGTTGSWGTA-3′ for the first round of amplification, and5′-ACMACIGTCCAYGCYIYCAC-3′and5′-TCRTACCASCARAYIAGYTT-3′ were used for the nested round of amplification. PCR conditions were the following: after initial denaturation for 5 min at 94°C, the reaction was subjected to 30 cycles of denaturation (94°C, 1 min), primer annealing (1 min), and extension (72°C, 1 min). The primer annealing temperature was 43 and 49°C in the first and second rounds of nested amplification, respectively. The amplification products were cloned into pGEMT-Easy (Promega). DNA sequencing was performed by the macromolecular sequencing facility at Brown University.

RT-PCR

RT-PCR without RNA isolation was performed as described in Klebe et al. (1996), with modifications. Briefly, a single sea urchin cell was washed three times in 1 M glycine pH 8.0 solution and lysed by freeze-thaw. Then, a single-step multiplex RT-PCR was carried out according to the manufacturer's directions using Access RT-PCR kit (Promega), with the addition of 1% DMSO and 1 M betaine to increase amplification efficiency. The RT reaction was performed for 45 min at 47°C; after denaturation for 5 min at 94°C, PCR amplification was performed for 45 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 2 min at 68°C. All reactions were performed in the linear range of amplification. Half of the sample was analyzed on a 2% agarose gel stained with ethidium bromide using digital camera and Gel Docker 1000 software. The amounts of primers used were 35 pmol per reaction of GAPDH primers, and 1 pmol per reaction of cyclin B primers. The sequences of the primers used for the cyclin B RT-PCR were 5′-GACAGCACCTTCTCT-

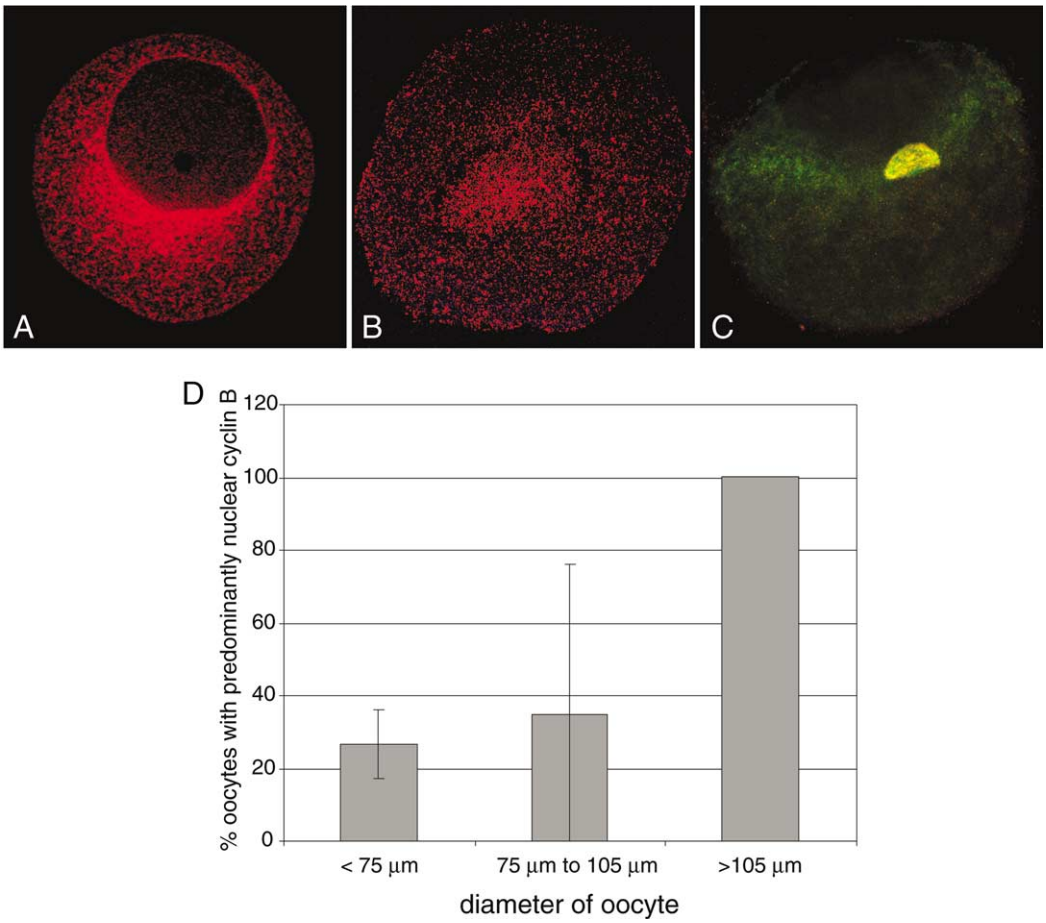

Fig. 6. Cyclin B localization in oocytes. Sea urchin oocytes were fixed in paraformaldehyde (all except (C)) or methanol (C) and immunostained with anti-cyclin B. (A) Immature oocyte, perinuclear cyclin B pattern. (B) Oocyte before GVBD, cyclin B is enriched in the germinal vesicle. (C) Meiotic metaphase, cyclin (in red) colocalizes with tubulin (green) on the meiotic spindle (yellow). (D) Increasing proportion of oocyte population exhibits nuclear enrichment of cyclin B protein as oocytes grow. Percentage of oocytes exhibiting a nuclear/cytoplasmic cyclin B ratio greater than 1 were determined for each size group. Shown are averages of values obtained in four separate experiments.

AGACG-3′ and 5′-TTGTCACTGTCGTCTTTGTCG-3′; primers for GAPDH were 5′-CACCCAGAAGATTGTT-GACGG-3′ and 5′-GACGAATGAGTTTGTAAGAGC-3′.

Results

Cyclin B cDNA analysis

L. variegatus cyclin B is a protein of 415 amino acids (predicted molecular weight 47.2 kDa; see Fig. 1). The protein encoded by the cDNA was identified as a cyclin B on the basis of similarity to the cyclin B family of proteins, especially in the cyclin box region (Fig. 1). Overall, sea urchin cyclin B showed the same extent of similarity to both the B1 and the B2 subtypes of vertebrate cyclins (and the same level of similarity to cyclins B4 and B5 of *Xenopus*), suggesting that it is not specified into either subtype (as is true for other echinoderm B-type cyclins; see Brandeis et al., 1998). This sea urchin cyclin B was the only cyclin B found in our screens and the only one found in genome and EST sequences currently available for this organism. In addition, evidence suggests only a single expressed form of cyclin B in yet another sea urchin, *S. purpuratus*, according to data generated by EST projects (http://sugp.caltech.edu).

The coding region of cyclin B transcript showed no sequence variation, in contrast to an earlier report of a cyclin B splice variant of another sea urchin, *Sphaerechinus granularis* (Lozano et al., 1998). However, the 5′UTR does have variability (Fig. 2). This was important for our functional analysis, since the morpholino oligonucleotides used for inhibition of protein synthesis needed to match the 5′UTR sequence exactly.

Expression and activity of cyclin B

Northern blot analysis detected a single cyclin B mRNA, approximately 5 kb long (Fig. 3A). Cyclin B mRNA levels were high in the ovary and unfertilized eggs and persisted

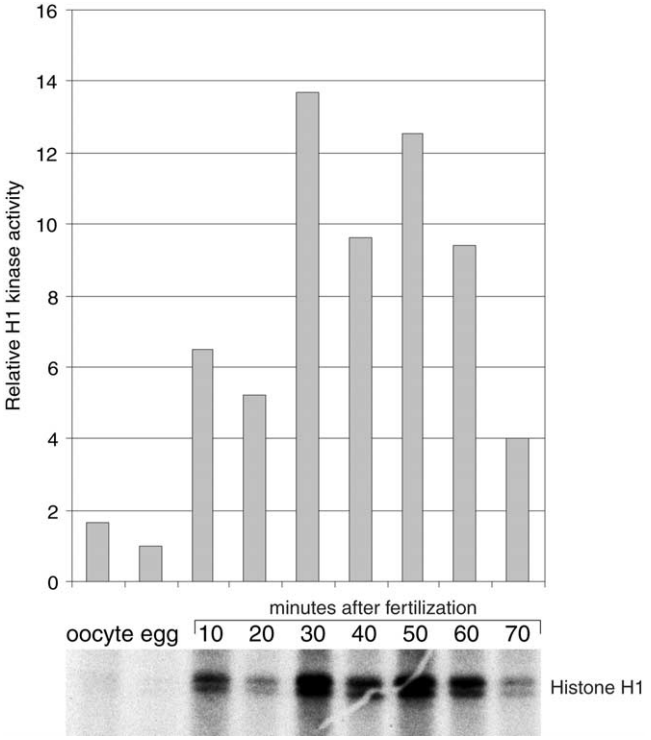

Fig. 7. Activity of cyclin B/cdk1 complex during development. Extracts prepared from *L. variegatus* oocytes, eggs, and embryos were immunoprecipitated with α-cyclin B and assayed for kinase activity using histone H1 as a substrate. Immunoprecipitation with an irrelevant antibody served as a control. Quantified values of kinase activities corrected for background are plotted above the respective lanes of the gel. The data shown are representative of three separate experiments.

throughout early development until the late blastula stage when they dropped precipitously. This is essentially in agreement with the cyclin B mRNA pattern reported for the sea urchin *Arbacia punctulata* (Pines and Hunt, 1987).

Using RNA in situ hybridization, we observed high levels of cyclin B mRNA in oocytes of all sizes, as well as in mature eggs (Fig. 3Bi and ii), while the ovarian tissue was mostly devoid of signal (Fig. 3Bii). Since even small premeiotic sea urchin oocytes (Fig. 3Bi) contain cyclin B mRNA, the regulation of cyclin B protein accumulation in these cells must occur at a posttranscriptional level. In embryos, the levels of cyclin B mRNA remained high throughout the early blastula stage, in agreement with the Northern blot data (Fig. 3Biii and iv), and the transcript was uniformly distributed throughout the cytoplasm of oocytes, eggs, and all cells of early embryos. In later embryos, cyclin B mRNA levels were slightly above the background in select tissues (gut and ciliary band; Fig. 3Bv). These same tissues are known to be still undergoing cell divisions and accumulating detectable levels of cyclin E mRNA (Sumerel et al., 2001).

We raised and affinity purified a polyclonal antibody against the C-terminal peptide of cyclin B. This antibody recognized a 49-kDa major band on immunoblots of sea urchin embryo lysates that approximates the molecular weight predicted for cyclin B (47.2 kDa) based on its amino acid sequence (Fig. 4). This specific immunoreactivity could be blocked by competition with the antigenic peptide (Fig. 4B). Surprisingly, the specific cyclin band was present not only during the G2/M phase as expected, but also in mature eggs, following meiosis (Fig. 4A). During the first cell cycle, cyclin B protein levels peaked at first metaphase (Fig. 4C), but a significant portion of cyclin B (60%) persisted throughout cytokinesis. Other cyclin B specific bands around 50 kDa were detected along with the major cyclin band, and their presence varied through the cell cycle. We conclude that these bands represent phosphorylated forms of cyclin since they are sensitive to phosphatase treatment (data not shown). These phosphorylated forms have been documented in many animals, including sea urchin (Meijer et al., 1989), goldfish (Yamashita et al., 1995), and *Xenopus* (Gautier and Maller, 1991). The phosphorylated form of cyclin was apparent on the immunoblot at 10 min after fertilization, much earlier than reported for *S. granularis* (Meijer et al., 1989, 1991), and remained until the onset of anaphase. Our data point to cyclin B being present in the cell much earlier than what has been detected by metabolic labeling of embryos with [^{35}S]methionine (Evans et al., 1983). In retrospect, this difference is not surprising, since metabolic labeling only detects newly synthesized protein, but it is unexpected, since we anticipated that cyclin B would be depleted in the egg following the final meiotic division.

Immunolocalization in situ was employed to address changes in cyclin B levels on a single-cell basis (Fig. 5). Cyclin B protein was detected in mature sea urchin eggs enriched in the pronucleus (Fig. 5A). Cyclin B was no longer detectable in the female pronucleus 10 min after fertilization (Fig. 5A, inset) and then it reentered the zygotic nucleus 35–40 min postfertilization. During mitosis, cyclin B concentrated on the mitotic spindle and following the initiation of anaphase, cyclin B disappeared from the spindle, starting in the middle and progressing toward the centrosomes (Fig. 5B–D). When the cells reached cytokinesis, cyclin B reaccumulated around the reformed nuclei (data not shown). The cytoplasmic cyclin B staining did not change significantly as embryos progressed from metaphase to telophase and into the next interphase, and it was not until after several divisions that the maternal, cytoplasmic cyclin B stockpile was depleted (Fig. 5E), consistent with the immunoblot data. Therefore, we conclude that total cyclin B levels exhibit less variance than expected during early sea urchin cell cycles, while it is local changes in abundance that regulate embryonic progression through mitosis.

Cyclin B protein was also abundant in developing oocytes. In midstage growing oocytes (less than 75 μm) cyclin B was localized to a perinuclear structure reminiscent of the endoplasmic reticulum (Fig. 6A). Cyclin B was seen in the nucleus (Fig. 6B) in some oocytes larger than 75 μm, and in most of the oocytes larger than 105 μm (Fig. 6D), corresponding to the size of mature eggs (which is 89.5 $\pm$ 9.5

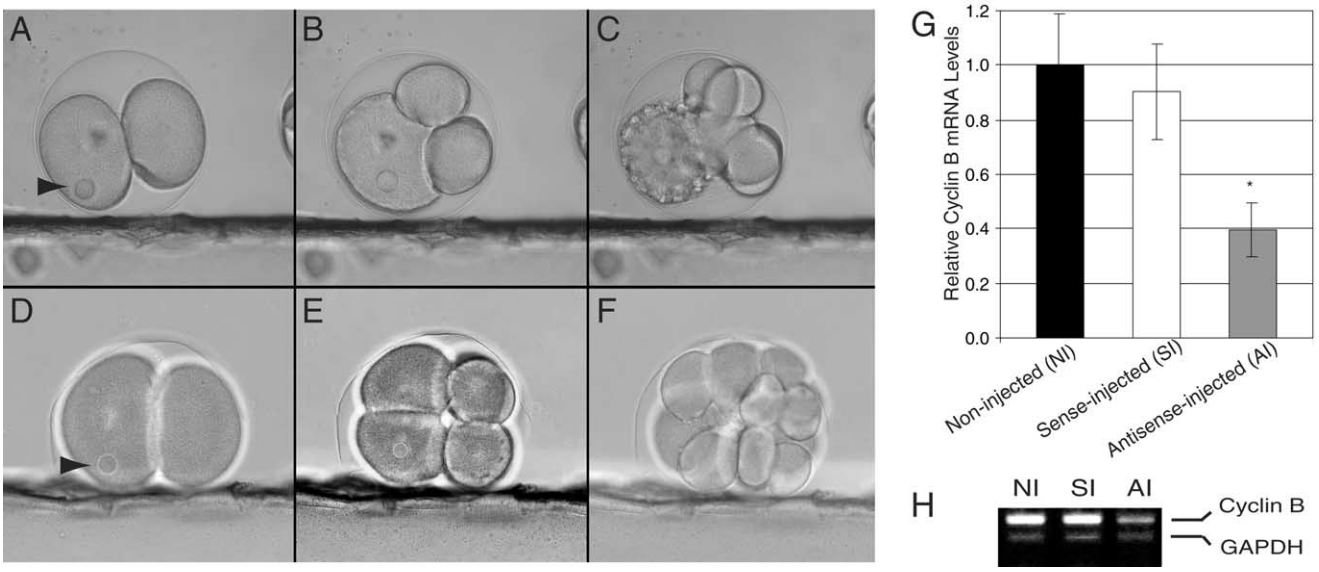

Fig. 8. (A–C) Injections of 25.9 μM DNA antisense ODN into one of the sister blastomeres results in the arrest of cell divisions and apoptosis, while the uninjected sister blastomere proceeds through cell divisions. (D–F) The control sense oligonucleotide injected blastomere undergoes normal cell divisions, as does the uninjected sister blastomere. Arrowheads: oil droplets mark the injected cells. (G) RT-PCR indicates that cyclin B mRNA is significantly degraded within 30 min following antisense treatment. Multiplex RT-PCR was performed on single one-cell zygotes that were injected with 25.9 μM of AS ODN, sense ODN, or left noninjected as a control. The ethidium bromide stained bands corresponding to cyclin B and GAPDH were quantified with Gel Docker 1000 software. Levels of cyclin B amplified product normalized to GAPDH product are represented. Shown are averages of four replicates of the experiment. The asterisk (*) indicates statistically significant difference from the control value ($P < 0.001$) by the Student's *t*-test. (H) A representative ethidium bromide stained gel with the results of multiplex RT-PCR.

μm in the population analyzed). After GVBD, cyclin B protein was enriched on the meiotic spindle microtubules (Fig. 6C), similar to what was seen on the meiotic spindles of starfish oocytes (Ookata et al., 1992, 1993).

To assess cyclin B associated kinase activity, we performed cyclin B immunoprecipitations followed by a histone H1 kinase assay (Fig. 7). We found that G2-arrested oocytes as well as postmeiotic eggs possessed low but detectable levels of H1 kinase activity. Following fertilization, the cyclin B associated H1 kinase activity increased significantly by 10 min in the first cell cycle, well in advance of mitosis, as seen previously in a different sea urchin, *S. granularis* (Geneviere-Garrigues et al., 1995). This activity further increased during mitosis before declining at anaphase. The decline of cyclin B associated kinase activity was incomplete, in agreement with partial depletion of cyclin B protein at that stage. Unfortunately, we could not similarly assess H1 kinase activity during an oocyte progression through meiosis because unlike frog or starfish oocytes, sea urchin oocytes enter meiosis spontaneously and asynchronously after removal from the ovary.

Synthetic requirements for cyclin B in embryonic divisions

To determine if cyclin B protein synthesis is necessary for oocyte maturation in sea urchins, we took two ap-

Table 1
Phenotypes of embryos that received injections of ODNs

Concentration of injected ODN[a]	ODN	Total embryos injected	Stopped division		Lagged behind uninjected		Wild-type phenotype
			Within the cell cycle of injection	In the next cell cycle following injection	At least 2 divisions behind uninjected	10–20 min delay relative to uninjected	
25.9 μM	AS	12	7	5	0	0	0
	S	14	1	0	0	6	7
5.2 μM	AS	13	0	0	4	7	2
	S	5	0	0	0	0	5

Note. The ODNs were injected into one of the sister blastomeres at indicated concentrations. The embryos were then followed under the microscope for 5 h. AS, antisense ODN; S, sense ODN.

[a] Final concentration of the injected compound in the cell.

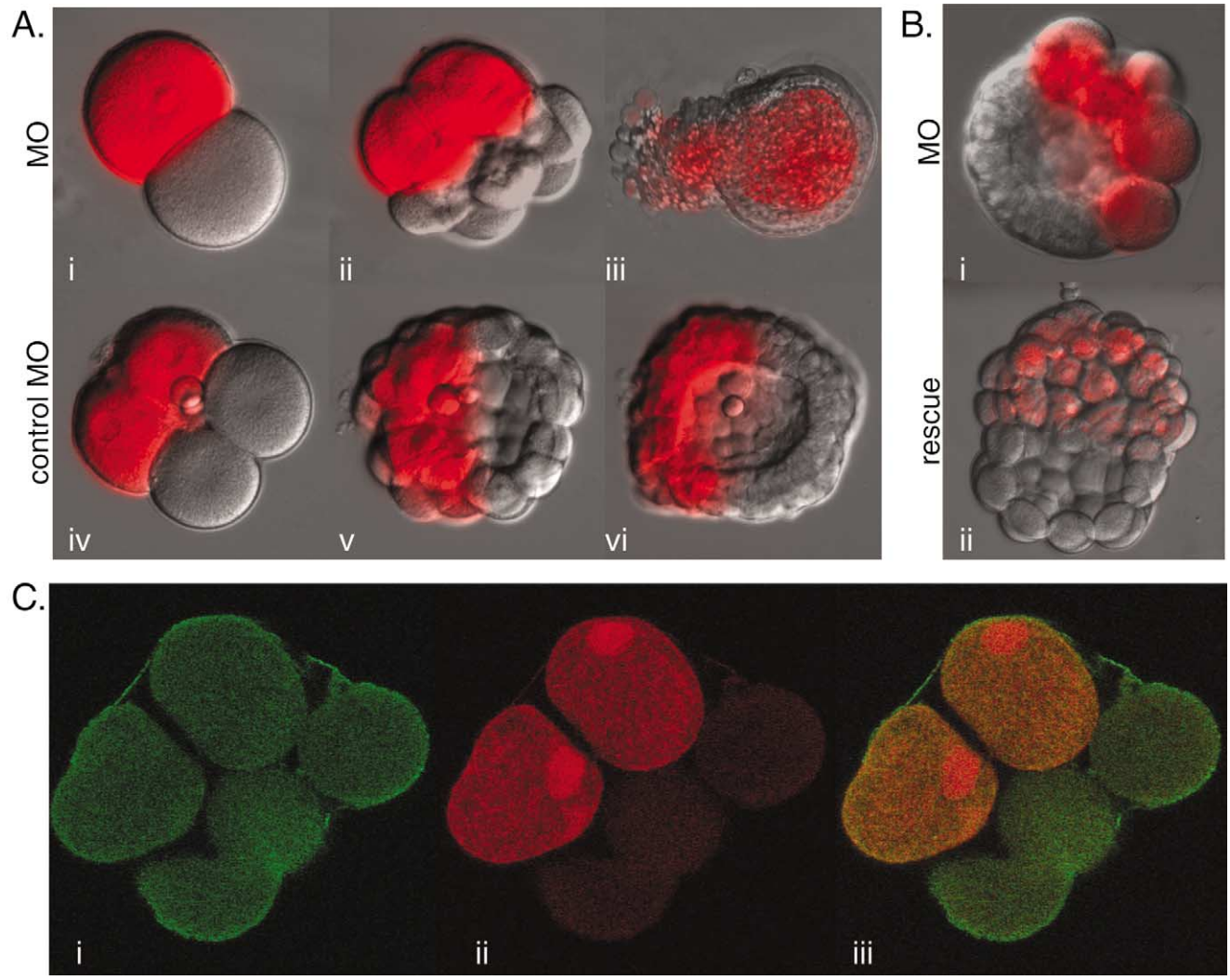

Fig. 9. Phenotype of embryos injected with cyclin B morpholino. (A) DIC images of MO2-injected embryos, overlaid with dextran epifluorescence to indicate which part of embryo received the morpholino (top) from two-cell (i) to "blastula" (iii); DIC images of embryos injected with control morpholino (bottom) from four cell (iv) to blastula (vi). (B) Rescue with cyclin RNA; (i) MO2-injected embryo half (marked with red dextran) lags behind the noninjected half; (ii) coinjection of polyadenylated cyclin B mRNA rescues the phenotype. (C) Cyclin B immunolabeling acquired by confocal scanning (i, pseudocolored green) is equal in injected (ii, dextran-marked pseudocolored red) and noninjected blastomeres; (iii) color overlay.

proaches of inhibiting de novo cyclin B synthesis: antisense oligodeoxynucleotides and morpholino oligomers. We first performed the analysis in embryos (since it is technically easier to ascertain the efficacy of the antisense knockouts), and then, in maturing oocytes.

We injected AS ODN in a single blastomere of a two-cell embryo and compared its cleavage phenotype with that of its sister blastomere. AS ODN injection (25.9 μM) caused arrest of early cleavage of the injected blastomere, while the control blastomere continued cell divisions (Fig. 8B). After missing two rounds of cleavage, the injected cell apoptosed (Fig. 8C) (Voronina and Wessel, 2001). This phenotype was not seen when the blastomere was injected with the same amount of sense ODN arguing for the specificity of the antisense effect (Fig. 8D–F). Microinjection of fivefold less antisense ODN had only reduced effects on cell divisions (see Table 1). Using RT-PCR, we determined that injection of 25.9 μM of the AS ODN in a one-cell zygote resulted in the degradation of ~60% of cyclin B mRNA within 30 min (Fig. 8G–H). Therefore, we attribute the observed phenotypes of the injected embryos to the decrease in cyclin B mRNA levels.

To achieve morpholino-mediated cyclin B protein depletion, we employed two morpholinos: MO1 and MO2 (Fig. 2). We designed MO1 based on our original cyclin B sequence, but subsequently found variability in the 5′UTR at MO1 binding site, with the major isoform representing approximately 80% of cyclin B mRNA population. We, therefore, designed MO2, a morpholino capable of binding to all species of cyclin B mRNA that we have found (see Fig. 2 for relative positions of MO1 and MO2). MO1 phenotypes were then regarded as resulting from 80% morpholino efficiency (Tables 2 and 4). Surprisingly, the phenotypes seen in morpholino-injected embryos differed from

Injected morpholino	Concentration	Total injected	Stopped divisions		Lagged behind noninjected half		Wild type
			within cell cycle of injection	in the following cell cycle	at least 2 divisions behind	less than 2 divisions behind	
Cyc B MO2	10 μM	8	-	-	100%	-	-
Cyc A MO	10 μM	8	12.5%	25%	50%	12.5%	-
Cyc B MO2 + Cyc A MO	10 μM each	4	25%	50%	25%	-	-
		8	12.5%	62.5%	25%	-	-

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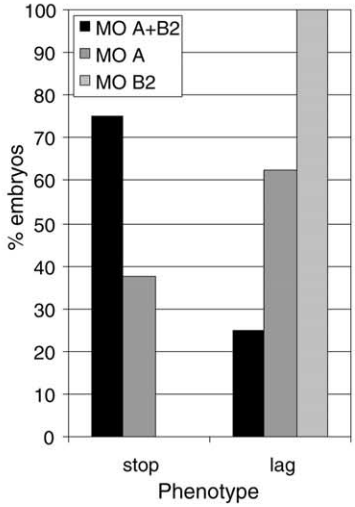

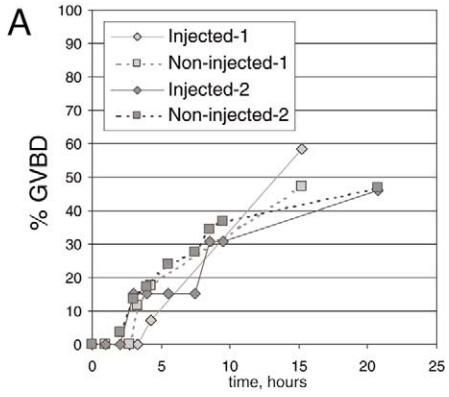

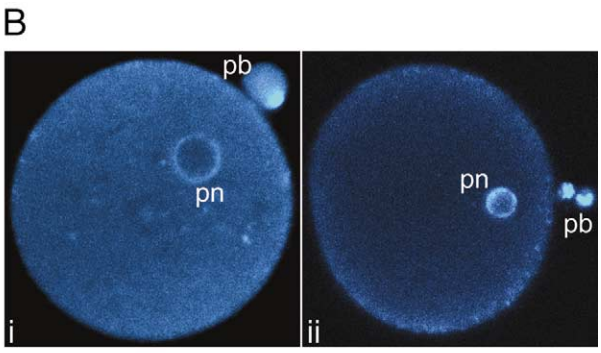

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Fig. 10. Cooperation of cyclins A and B in the regulation of embryonic divisions. One blastomere of a two-cell embryo was injected with the indicated morpholino oligo (or mix containing 10 μM of each MO). Progression of the injected blastomere through the cell cycle was compared with that of the control sister blastomere. Results are displayed in table and bar graph format.

Fig. 11. (A) Time course of the induction of oocyte maturation. Injection of cyclin B MO1 at 300 μM concentration or MO2 at 10 μM concentration did not result in retardation of spontaneous entry of the oocytes into meiosis, when compared with control oocytes. (B) MO2-injected oocyte forms a cell with an enlarged pronucleus, and a single polar body (i), significantly different from the control in vitro matured egg (ii). pn, pronucleus; pb, polar body.

those of antisense ODN injected ones. Morpholino-injected blastomeres never ceased early cell divisions, although they lagged significantly behind the uninjected sister blastomeres (Fig. 9). Only extremely high concentrations of MO2 (300–500 μM) succeeded in blocking cell-cycle progression (see Table 2). However, MO injections appeared to induce a later phenotype: the cells exposed to 20 μM or more of MO1 or 2 stopped divisions and failed to be integrated into the whole embryo at the late blastula (see Fig. 9A and Table 2). This effect was concentration-dependent and may reflect an eventual depletion of maternal cyclin B stocks.

To address whether the observed phenotypes were due to reduction in cyclin B protein levels, we fixed the injected embryos 2 h after injection and performed immunolocalizations with anti-cyclin B antibodies. Immunolocalizations detected cyclin B protein still present in both MO1- and MO2-injected cells in amounts comparable to those in control cells (MO1: data not shown; MO2: Fig. 9C). We interpreted this result to mean that the substantial amounts of maternal cyclin B present in eggs were not depleted during the course of this experiment. To test whether the observed phenotypes in fact resulted from inhibition of de novo cyclin B synthesis, we assessed if they would be rescued by coinjection of cyclin B morpholino-insensitive mRNA along with the morpholino. Coinjection of 17.3 μg/ml final concentration of polyadenylated cyclin B mRNA with MO2 completely rescued the morpholino-induced lagging phenotype in 63% of injected embryos, while the remaining 37% embryos showed significant alleviation of the phenotype (see Table 2 and Fig. 9B). Therefore, the observed phenotypes appear to result from selective inhibition of cyclin B synthesis, which may not be essential until the late stages of development when the maternal stock of cyclin B protein is depleted. The difference in phenotype resulting from AS ODN and MO (death vs delay) likely represents an additional toxicity resulting from effective concentrations of ODN (Dupre et al., 2002; Heasman, 2002).

Table 2
Phenotypes of embryos that received morpholino injection

Injected moropholino	Concentration within injected cell	Total injected	Stopped divisions		Lagged behind noninjected half		Wild-type	After 21 h		
			Within cell cycle of injection	In the following cell cycle	At least 2 divisions behind	Less than 2 divisions behind		Total	Wild-type	Deformed
CycB MO1	2 μM	4	—	—	—	100%	—	0		
	6.4–11.2 μM	7	—	—	100%	—	—	0		
	21.9 μM	18	—	—	100%	5%	—	5	40%	60%
	100 μM	4	—	—	100%	—	—	4	—	100%
	200 μM	5	—	60%	40%	—	—	5	—	100%
	300 μM	8	—	—	100%	—	—	8	—	100%
CycB MO2	10 μM	8	—	—	100%	—	—	0		
	100 μM	8	—	—	88%	12%*	—	0		
	200 μM	5	—	40%	60%	—	—	0		
	300 μM	9	22%	—	78%	—	—	3	—	100%
			37%	—	63%	—	—	0		
	500 μM	4	25%	25%	50%	—	—	0		
CycB MO1 + CycB MO2	100 μM each	5	20%	—	60%	20%*	—	1	—	100%
CycB MO2 + cyclin mRNA	10 μM MO	8	—	—	—	37.5%	62.5%	3	100%	
Inverted CycB MO1	21.9 μM	4	—	—	—	—	100%	4	100%	—
	53.2 μM	6	17%	—	—	34%	49%	4	100%	—
	300 μM	7	—	—	—	14%	86%	5	60%	40%
	500 μM	7	—	—	14%	14%	72%	5	20%	80%

Note. The indicated morpholinos were injected into one of the sister blastomeres at concentrations noted. The embryos were then followed under the microscope for 20 h. The positions of morpholinos 1 and 2 on cyclin B mRNA are noted in Fig. 2.

* Both injected and noninjected halves were slow relative to noninjected embryos.

Cyclin A compensates for cyclin B loss in the embryos

Another possible explanation for the morpholino-induced phenotype in embryos is compensation by cyclin A protein that is able to activate the same kinase, cdk1. The "lagging" phenotype observed in our studies was clearly reminiscent of *Drosophila* cyclin B knockout embryos that are able to proceed, although slower than the wild-type ones, through embryonic divisions even after exhausting the maternal cyclin B protein stores (Knoblich and Lehner, 1993). Therefore, we tested whether mitotic cyclins in the sea urchin could compensate for each other. Injection of 10 μM cyclin A morpholinos into one of the sister blastomeres led to cessation of embryonic cell cycles in 35% of cases, with an exaggerated phenotype induced by coinjection of 10 μM cyclin B morpholino (Fig. 10). To test whether synthesis of either mitotic cyclin was required for the first zygotic division as suggested by inhibition of this division by protein synthesis inhibitor emetine (Wagenaar, 1983), we injected unfertilized eggs with individual morpholinos against cyclins A and B or a combination thereof (Table 3). When injected prior to fertilization, 40 μM cyclin A morpholino blocked cell-cycle progression in 80% of embryos, while 40 μM cyclin B morpholino was only 17% efficient by this criterion. The combination of morpholinos was more effective than either one on its own, blocking division in 100% of zygotes. These results suggest a functional overlap between cyclins A and B during early embryonic divisions and that embryonic synthesis of (predominantly) cyclin A protein is required for the first division even though the egg is endowed with significant maternal cyclin B protein stores.

Table 3
Cyclin A protein synthesis is required for the first mitotic division

Injected morpholino	Concentration	Total zygotes	Blocked divisions
Cyc B MO2	40 μM	6	17%
Cyc A MO	40 μM	5	80%
CycB MO2 + CycA MO	40 μM each	11	100%

Note. Mature sea urchin eggs were injected with the indicated concentration of morpholino (against cyclin A, cyclin B, or both) and were then fertilized after 90-min incubation period. The ability of the injected zygotes to complete first mitotic division was assessed at 90 min postfertilization (normal time of the first division in this species under our conditions is 70 min).

Table 4
Influence of MO injection on oocyte maturation

Morpholino injected[a]	Total number of oocytes	Resting oocytes		GVBD[b]		"Mature eggs"	
		%	Average	%	Average	%	Average
Cyclin B	12	41.7	47.6%	16.7	16.1%	41.7	36.3%
MO1, 300 μM	13	53.8		15.4		30.8	
Cyclin B	9	33.0	44.3%	22.3	22.2%	44.4	33.5%
MO2, 10 μM	9	55.6		22.2		22.2	
Cyclin A MO	4	50.0	42%	0.0	8%	50.0	50%
10 μM	8	37.5		12.5		50.0	
Cyclin A MO + Cyclin B MO2	9	44.5	44.5%	33.3	33.3%	22.2	22.2%
Noninjected (control)	17	52.9	53.1%	0	5.0%	47.1	41.9%
	30	53.3		10.0		36.7	

Note. Assessment of oocytes' progress through meiotic maturation 20 h postinjection with indicated reagents.
[a] Final concentration of the injected compound in the cell.
[b] GVBD, Cells that have undergone GVBD, but have not reformed the pronucleus or died.

Cyclin B synthesis is necessary for completion of meiotic maturation

Full-grown oocytes of the sea urchin will enter meiosis when isolated from the ovary (Berg and Wessel, 1997), but unfortunately, we currently have no experimental control over the timing or synchrony of this process. Sea urchin oocytes injected with the highest concentration of cyclin B MO1 did not exhibit any noticeable phenotypic difference from noninjected or inverted MO-injected control groups. Oocytes injected with cyclin B MO2 underwent GVBD at the same rate as the control groups of oocytes (Fig. 11A, and Table 4). However, the progression of MO2-treated oocytes through meiosis was not normal. The MO2-injected oocytes (100%) extruded only one polar body instead of two before re-forming the pronucleus, suggesting that they exited the meiotic cell cycle after MI. The formed polar body was nearly twice as large as those of the control in vitro matured eggs, and the pronucleus detected in MO2-injected cells appeared enlarged (20 μm diameter on average) in comparison to those of the normal eggs (15 μm diameter on average; Fig. 11B). Furthermore, the amount of DNA in the pronuclei of MO-injected cells as assessed by the intensity of Hoechst staining was on average twice or more (250% on average) the amount of DNA in control in vitro matured eggs (data not shown). This suggests that these nuclei are diploid, not haploid, and lends further support to the conclusion that these cells have only undergone one round of meiotic divisions. We conclude that sea urchin oocytes do not require cyclin B synthesis for entry into meiosis and GVBD, but do require cyclin B synthesis for meiotic completion and normal progression to a mature egg.

As an alternative approach to inhibiting cyclin B synthesis, we investigated the maturation of sea urchin oocytes exposed to solutions of AS ODN directed against cyclin B. The results of the antisense treatment were very similar to those achieved with MO2. Exposure to 10 μM AS ODN solution did not prevent the treated groups of oocytes from entering meiosis to the same extent as control groups cultured in ASW or 10 μM sense ODN (see Table 5). However, fewer of the AS-treated cells matured (contained pronucleus) than in control groups. Since sea urchin oocytes enter meiosis spontaneously and asynchronously, we were not able to effectively follow the progression of populations of cells through maturation to identify specific stages of meiotic progression. Instead, we performed several phenotypic analyses on individual AS ODN-treated cells. These include staining with Hoechst (for DNA), and staining for cortical granules to assess the state of cytoplasmic maturation (Berg and Wessel, 1997). The nuclear stain revealed

Table 5
Phenotype of oocytes treated with ODN

Medium	Total number of oocytes	Resting oocytes		GVBD[a]		Mature eggs		Dead cells	
			%		%		%		%
10 μM, antisense ODN	33	10	30	6	18	4	12	13	40[b]
10 μM, sense ODN	32	10	31	4	13	12	37[b]	6	19
Sea water (control)	31	11	36	2	6	14	46[b]	4	12

Note. The oocytes were incubated in the solution of antisense ODN, sense ODN, or ASW, as described under Materials and Methods. The meiotic progression and state of the population was assessed after 19 h in culture.
[a] GVBD, Cells that have undergone GVBD, but have not reformed the pronucleus or died.
[b] Highlighted are the categories that were most prevalent in each group.

that the pronuclei of the antisense-treated cells appeared enlarged in comparison to the control ones, consistent with the phenotype of MO2-injected cells (data not shown). Therefore, it is possible that the cells treated with antisense cyclin B ODN could not normally execute meiosis. Cortical granules were found translocated to the surface of the antisense-treated cells as in control groups (data not shown). We conclude that the initial rise in MPF activity predicted at GVBD is sufficient for completion of cortical granule translocation; however, nuclear maturation (meiosis) is incomplete following cyclin B antisense treatment.

Cyclin A protein synthesis is not necessary for meiotic maturation

Since we detected a functional overlap of cyclins A and B in the early embryonic cycles, we hypothesized that this overlap could also operate during oocyte meiotic maturation and explain why entry into meiosis (or GVBD) was unaffected by cyclin B MO. To test this hypothesis, we injected the oocytes with 10 μM cyclin A morpholino, the concentration that proved effective in the embryos. These oocytes proceeded through meiotic divisions indistinguishably from the control ones and formed mature eggs with pronuclei (see Table 4, and data not shown). Furthermore, injection of a combination of cyclin B and cyclin A morpholinos produced a phenotype identical to that of cyclin B morpholino alone (i.e., normal entry into meiosis, followed by reformation of enlarged pronucleus after the first meiotic division; see Table 4, and data not shown). Since we know that cyclin A mRNA and protein are present in these oocytes (E.V. and G.M.W., unpublished observations), our data agree with evidence from clam and *Xenopus* oocytes that cyclin A synthesis is not required for meiotic maturation (Hunt et al., 1992; Minshull et al., 1991).

Discussion

Sea urchin eggs are used extensively for fertilization studies, and their early embryos were instrumental in the identification of cyclins. Yet, regulation of meiosis in this animal is largely unknown because of the lack of knowledge in handling and working with their oocytes. We have attempted to overcome some of these shortcomings and examine how the oocyte proceeds through meiosis, since it is one of the few opportunities to study oocytes that complete meiosis before fertilization.

Cyclin B, a ubiquitous regulator of M-phase progression, is regulated in diverse ways even in the same cell type. These differences are especially apparent in the regulation of an oocyte's entry into meiosis, where MPF can be activated at multiple levels, such as translational regulation as in fish (Yamashita, 1998) or posttranslational modifications and subcellular localization as in *Xenopus* (Hochegger et al., 2001). We find that sea urchin oocytes do not need to make more cyclin B protein for entry into meiosis (GVBD), yet they do need to make more cyclin B for normal M-I spindle formation and polar body extrusion. We confirm that these oocytes also need to make cyclin B to escape interphase between two consecutive M-phases, as in other animals. Furthermore, we find that the postmeiotic sea urchin egg contains significant maternal cyclin B that is used during the rapid early cleavage divisions. Our data identify significant differences between the regulation of meiotic and mitotic divisions in the sea urchin and modifies our perspective on the metabolism and role of cyclin B protein from its first description in this organism.

Mitotic cyclins in sea urchin oocyte maturation

Our work provides the first direct molecular evidence of specific requirement for cyclin B synthesis in the first meiotic division of echinoderms. We have found cyclin B localized to the spindle microtubules in sea urchin maturing oocytes at metaphase, at the right place and the right time to function in the regulation of the spindle dynamics. Cyclin B has been found to regulate microtubule dynamics in mitotic *Xenopus* extracts (Verde et al., 1992), as well as spindle formation in *Drosophila* embryos (Jacobs et al., 1998; Knoblich and Lehner, 1993; Stiffler et al., 1999) and meiotic spindle formation and polar body extrusion in frog *Rana japonica* (Kotani et al., 2001). Elongation of meiotic spindles (similar to mitotic spindles) caused by reduction in cyclin B levels may provide a mechanistic explanation for our observed enlargement of the first polar bodies (a longer spindle results in extrusion of a larger cell fragment). The mechanism of MPF activation in sea urchin is presently not known. We do know, however, that the mitogen signal for maturation is different than in starfish since 1-methyladenine, which induces meiosis in starfish oocytes, is inactive in the sea urchin.

Sea urchin eggs contain significant amounts of cyclin B

The analysis of cyclin B protein expression and synthetic requirements during early embryogenesis of *L. variegatus* yielded unexpected results: the egg has sufficient maternal cyclin B stores to support the first cell cycle. A wealth of data documents the requirement for general protein synthesis in order for the increase in H1K activity and cell division during the first cell cycle of sea urchin embryos of various species (Arion and Meijer, 1989; Meijer and Pondaven, 1988; Meijer et al., 1991; Wagenaar, 1983). However, specific requirements for cyclin B synthesis were rarely addressed. Mature sea urchin eggs accumulate cyclin B before fertilization (our data and Moreau et al., 1998). Even though we have detected an increase in cyclin B levels from the egg to the metaphase embryo (in agreement with Evans et al., 1983 and others), it occurs well after the reported emetine-sensitive window (at 70 min vs 15; Wagenaar, 1983), and

this increase is largely dispensable for the first cell division (Table 3).

Functional overlap between cyclins A and B is present only at certain stages

We found that mitotic cyclins A and B partially compensated for each other in early sea urchin embryos. This overlap is similar to that reported in *Drosophila* embryos, where single knockouts of mitotic cyclins A, B, or B3 were able to proceed through divisions longer than either of the double knockouts (Edgar et al., 1994; Jacobs et al., 1998; Knoblich and Lehner, 1993). This compensation is not complete, however, so each individual cyclin is still essential, yet these cells can tolerate significant reductions in amounts of one of the proteins. This functional overlap was less pronounced during the first mitotic division, where zygotic synthesis of cyclin A was required in 80% of the embryos to go through first cell cycle (Table 3).

Our data suggest a lack of cooperation between mitotic cyclins A and B in the regulation of oocytes' meiosis. This is intriguing, since there are very few studies as to whether the functions of different M-phase cyclins are distinct during meiosis. The data from *Drosophila* suggest that cyclins B and B3 are individually essential for oogenesis, implying that functional overlap between mitotic cyclins at this stage is less pronounced (Jacobs et al., 1998). On the other hand, an example of compensation between mitotic cyclins is the maintenance of the MI arrest before fertilization in oocytes of the mollusk *Patella vulgata* (van Loon et al., 1991). In contrast, both mitotic cyclins are required for the meiotic G2/M transition in mouse spermatogenesis, where targeted deletion of cyclin A1 gene does not impair mouse viability, but prevents activation of cyclin B (1 and 2)–cdk1 complexes at the G2/M transition of sperm meiosis (Liu et al., 2000).

Distinct types of cell-cycle regulation

The cell cycles of metazoan oocytes and early embryos are substantially distinct from those of later embryos and somatic cells. These cell cycles differ in the relative lengths of their respective phases, and activity of checkpoint controls. Accordingly, the patterns and status of molecular cell-cycle regulators expression differ dramatically between these cell types as well. For example, G2-arrested oocytes of many species do not contain cyclin A protein, while the early embryos do (for example, reviewed in Kishimoto, 1999). Furthermore, meiosis in clam and *Xenopus* oocytes does not require cyclin A protein (Hunt et al., 1992; Minshull et al., 1991), although it can be induced by introduction of exogenous cyclin A (Swenson et al., 1986). In contrast, fish oocytes have cyclin A protein, but the cyclin A/cdk1 complexes normally become active only late in meiosis, and premature activation of cyclin A/cdk1 does not induce GVBD (Katsu et al., 1995). Together with our results suggesting lack of compensation by cyclin A for the reduction in cyclin B levels during meiosis in sea urchin oocytes despite the presence of cyclin A mRNA and protein in the oocyte (data not shown), this evidence implies a lack of meiotic MPF function for cyclin A. A switch in the repertoire of molecular cell-cycle regulators has also been documented in animals at the midblastula stage, for example, in *Xenopus* (Hartley et al., 1996) and in sea urchins (Moore et al., 2002). Thus, the observed differences in requirements for cyclin B synthesis for cell-cycle progression between early and late embryonic stages of the sea urchin may be caused by transitions in the mechanisms of cell-cycle control in later embryonic cells.

Acknowledgments

We thank past and present members of PRovidence Institute of Molecular Oogenesis for unwavering support and assistance, especially Emma R. Green for help in cloning sea urchin GAPDH, Dr. Jacqueline M. Brooks for aid in developing the RT-PCR technique, and Audrey S. Howell for contribution of an EST clone spanning cyclin B 3′ UTR. Our gratitude extends to Dr. Bradley J. Schnackenberg for supplying protocols, reagents, and assistance in performing histone H1 kinase assays, as well as to Dr. Bettina Meier, Julie A. Boerckel, and Özlem Yildiz for help in performing the activity assays. We appreciate special assistance of Wayne Baumgartner of CCAT. This research was supported by NIH and NSF grants to G.M.W.

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